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Isolation of an RNA-Directed RNA Polymerase–Specific cDNA Clone from Tomato

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A 3600-bp RNA-directed RNA polymerase (RdRP)–specific cDNA comprising an open reading frame (ORF) of 1114 amino acids was isolated from tomato. The putative protein encoded by this ORF does not share homology with any characterized proteins. Antibodies that were raised against synthetic peptides whose sequences have been deduced from the ORF were shown to specifically detect the 127-kD tomato RdRP protein. The immunoresponse to the antibodies correlated with the enzymatic activity profile of the RdRP after chromatography on Q-, poly(A)–, and poly(U)–Sepharose, hydroxyapatite, and Sephadex G-200 columns. DNA gel blot analysis revealed a single copy of the *RdRP* gene in tomato. RdRP homologs from petunia, Arabidopsis, tobacco, and wheat were identified by using polymerase chain reaction. A sequence comparison indicated that sequences homologous to RdRP are also present in the yeast *Schizosaccharomyces pombe* and in the nematode *Caenorhabditis elegans.* The previously described induction of RdRP activity upon viroid infection is shown to be correlated with an increased steady state level of the corresponding mRNA. The possible involvement of this heretofore functionally elusive plant RNA polymerase in homology-dependent gene silencing is discussed.

INTRODUCTION

RNA-directed RNA polymerase (RdRP) from healthy tomato leaf tissue seems to represent a plant-specific and hence exceptional nucleic acid–synthesizing enzyme because higher plants are the only eukaryotes in which the presence of a cellular RdRP has been unambiguously demonstrated to date (for discussion, see Schiebel et al., 1993a, 1993b). RdRP activity has been detected in Chinese cabbage (Astier-Manifacier and Cornuet, 1971), cauliflower (Astier-Manifacier and Cornuet, 1978), tobacco (Duda et al., 1973; Duda, 1979; Takanami and Fraenkel-Conrat, 1982), tomato (Boege and Sänger, 1980), cowpea (Dorssers et al., 1982), and cucumber (Khan et al., 1986), but only the RdRP from tomato leaf tissue has been isolated and characterized with respect to its physicochemical (Schiebel et al., 1993a) and in vitro catalytic (Schiebel et al., 1993b) properties. These cellular RdRPs should not be mistaken for RNA-dependent RNA polymerases (EC 2.7.7.48), which become detectable when bacteria and eukaryotes are infected with RNA viruses. RNA-dependent RNA polymerases mediate viral RNA replication and are therefore much more appropriately called virus RNA replicases.

Despite all of these studies, the origin and the actual biological function(s) of plant-encoded RdRP have remained unresolved and are enigmatic because its cognate template(s) and in vivo transcription products remain unknown. Nevertheless, we surmised (Schiebel et al., 1993b) that in the cell, RdRP might be of paramount importance because it transcribes from corresponding RNA sequences small RNA molecules that control the synthesis of nucleic acids and their translation into proteins.

Studies on the induction of a highly specific antiviral state in transgenic plants led to a hypothesis that cellular RdRP could play a role in post-transcriptional gene silencing (Lindbo et al., 1993). Post-transcriptional gene silencing was thought to involve an RNA-dependent process that degrades (trans)gene-specific mRNA in the cytoplasm (reviewed in Baulcombe, 1996; Depicker and Van Montagu, 1997; Stam et al., 1997a; Wassenegger and Pélissier, 1998). Numerous examples have been reported in which the introduction of a transgene that encodes part or the entire sequence of a host gene can lead to cosuppression of all of the transgene and homologous host gene copies (Napoli et al., 1990; Smith et al., 1990; van der Krol et al., 1990; de Carvalho et al., 1992; Dorlhac de Borne et al., 1994; van Blokland et al., 1994; de Carvalho Niebel et al., 1995; Vaucheret et al., 1995). Post-transcriptional inactivation of

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foreign transgenes that are not homologous to host genes has also been reported (Hobbs et al., 1990, 1993; Dehio and Schell, 1994; Ingelbrecht et al., 1994; Elmayan and Vaucheret, 1996). In addition, several examples of plant resistance against infectious RNA viruses that display homology to a post-transcriptionally silenced transgene appeared to be mediated by the same cytoplasmic mechanism that is responsible for the disappearance of the (trans)gene mRNA (Lindbo et al., 1993; Smith et al., 1994; English et al., 1996; Sijen et al., 1996).

To account for the sequence specificity of post-transcriptional gene silencing, it has been suggested that the degradation mechanism could be specifically mediated via short complementary RNAs (cRNAs) synthesized from the transgene RNA by a cellular RdRP (Dougherty and Parks, 1995). Interaction between sense RNA molecules and cRNAs would lead to the formation of double-stranded RNA (dsRNA) structures. The recognition of the duplex structure by dsRNA-specific RNases could then represent the initial step in mRNA degradation.

During the past few years, the phenomenon of homology-dependent gene silencing has been extensively studied, and several attempts have been made to combine all observations into working models (Dougherty and Parks, 1995; Meyer, 1995; Baulcombe, 1996; Baulcombe and English, 1996; Prins and Goldbach, 1996; Sänger et al., 1996; Sijen et al., 1996; Depicker and Van Montagu, 1997; Stam et al., 1997a; Wassenegger and Pélissier, 1998). Although these models differ in some major aspects, a central role for plant RdRP-produced cRNAs in the degradation mechanism is intrinsic to all of these models. This is remarkable because convincing evidence for the existence of such RdRP-synthesized cRNAs is still missing.

The most promising approach to obtain direct experimental evidence for an RdRP-mediated RNA degradation process would be the availability of the RdRP itself as well as the cloning of its cDNA. By using an active enzyme, one could analyze RdRP substrate specificity in vitro. Transformation experiments with RdRP-specific sense and antisense cDNA constructs might result in plants expressing increased and decreased enzyme activities, respectively. Substantial changes in the occurrence of post-transcriptional gene silencing in such lines, as compared with plants displaying wild-type RdRP expression, could provide evidence for the crucial role of this enzyme in gene silencing.

In this study, we report the foundation on which these future studies can be built, namely, the isolation of a full-length cDNA that encodes the 127-kD tomato RdRP. The complete cDNA sequence of the *RdRP* gene, its genomic organization in tomato, and evidence for the presence of the RdRP sequences in four additional higher plants are presented. On the basis of our sequence data, we discuss the likely possibility that there are RdRP homologs in non-plant species, such as in the yeast *Schizosaccharomyces pombe* and in the nematode *Caenorhabditis elegans.* Experimental evidence for a correlation between the cDNA-encoded protein (C-RdRP) and the tomato RdRP (T-RdRP) is provided and is based on immunodetection analysis of the tomato leaf enzyme by using antibodies that were raised against cDNA-specific peptides. Finally, an improved preparation procedure for the isolation of active T-RdRP from leaf tissue is described.

RESULTS

Purification of RdRP for Microsequencing

We have observed that RdRP activity is increased not only in virus-infected plants, but also in viroid-infected tomato (Schiebel et al., 1993a). However, because viroids do not encode proteins, no viral RNA replicases were detected in viroid-infected plants. Consequently, any RdRP activity detectable in these plants was due solely to the host-encoded enzyme. This situation facilitated the isolation of the T-RdRP. Its activity was induced up to fivefold, and for each purification step, the recovered activity could be clearly assigned to the tomato enzyme. Nevertheless, to yield sufficient amounts of protein for microsequencing, we had to process 450 g of viroid-infected apical tomato leaves, essentially as described by Schiebel et al. (1993a) (see Methods). In our previous study, we reported that RdRP fractions that had been eluted from a DEAE–Sepharose column were subjected to affinity chromatography on dsDNA–cellulose (Schiebel et al., 1993a). However, in later experiments, we failed to obtain highly active enzyme preparations when newly prepared or commercially available dsDNA–cellulose preparations were used. Therefore, the dsDNA–cellulose chromatography and the subsequent MonoQ purification step were substituted with two rounds of poly(U)–Sepharose chromatography. After these steps, ~20 units (50 pmol) of partially purified T-RdRP protein were collected; after SDS-PAGE, the 127-kD protein was used for the microsequencing procedure (described in Methods).

Isolation and Cloning of RdRP-Specific Sequences

Endoproteolytic digestion of the gel-excised 127-kD protein(s) resulted in ~50 reversed-phase HPLC–specific peaks. Most of these contained a mixture of several protein degradation products. Nevertheless, microsequencing of peptides of the T-RdRP resulted in four amino acid sequences that could be used to design degenerate primers. The sequence of two of the four peptides and the sequence of four of the corresponding synthetic oligonucleotides that had been designed for polymerase chain reaction (PCR) experiments are presented in Figure 1.

Because the relative positions of the sequenced peptides within the T-RdRP were not known, we performed PCR with tomato cDNA by using primer pairs A and B, respectively

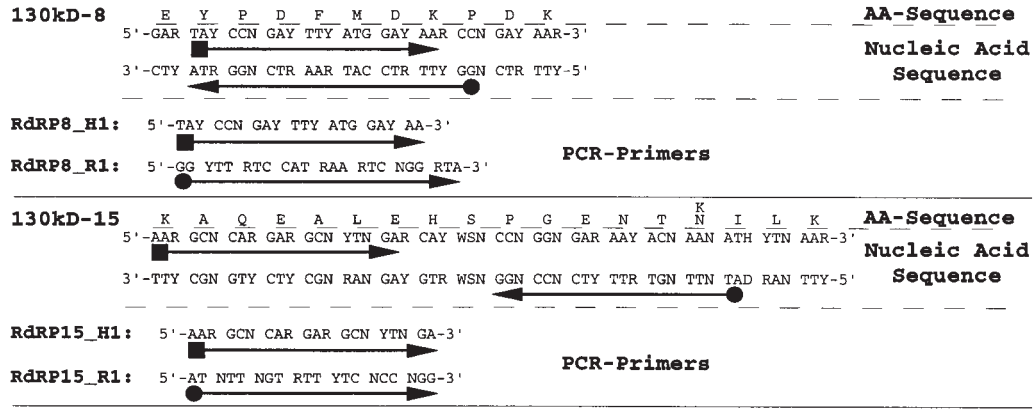


Figure 1. Design of RdRP-Specific PCR Primers.

Four PCR primers (RdRP8_H1, RdRP8_R1, RdRP15_H1, and RdRP15_R1) were deduced from the amino acid (AA) sequences of two (130kD-8 and 130kD-15) of the four peptides that were recovered after microsequencing of the T-RdRP enzyme. Primer pair A (RdRP8_H1/RdRP15_R1) and primer pair B (RdRP15_H1/RdRP8_R1) were used in PCRs with tomato cDNA.

(see Figure 1). Analysis of the PCR products revealed that a single DNA fragment of ~800 bp had been amplified with primer pair B. This fragment ($RdRP_{800}$) was cloned into pT-PCR and sequenced. As shown in Figure 2, the precise length of $RdRP_{800}$ was 833 bp, comprising an open reading frame (ORF) of 277 amino acid residues.

Two ZAP Express EcoRI cDNA libraries were screened three times with the $RdRP_{800}$ DNA fragment (see Methods), and 23 recombinant plasmid DNAs that had been detected with the radioactive probe were excised. Characterization of the plasmid inserts revealed that none of them corresponded to the mininum length of ~3.0 kb, which would be expected for a 127-kD protein. The largest hybridizing EcoRI cDNA fragment (RdRP24) was ~2.3 kb and contained the entire 3′ part of an RdRP-specific cDNA.

To obtain the missing 5′ region of the RdRP cDNA, we performed rapid amplification of 5′ cDNA ends (5′ RACE) using the GSP400/AP1 primer pair (see Methods). This allowed the amplification of a major product of ~1.9 kb. Computer-supported analysis of the sequence resulting from the overlapping $RdRP_{5'\ RACE}$ and the RdRP24 clones revealed (1) the entire cDNA sequence (Figure 2A); (2) the sequence of an ORF of 3.345 kb, which corresponds to a predicted protein (C-RdRP) comprising 1114 amino acids (Figure 2B); and (3) a calculated molecular mass of the C-RdRP of ~127 kD, which is in good agreement with the experimental values that had been determined for the T-RdRP by using SDS-PAGE (128 kD) and sucrose gradient centrifugation (119 kD) (Schiebel et al., 1993a).

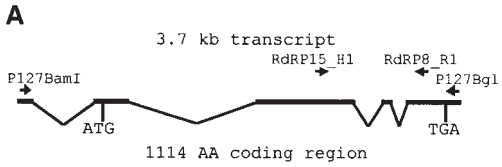
To obtain a nearly full-length RdRP cDNA clone, we performed PCR amplification using the primer pair P127BamI/P127Bgl (Figure 2A). By characterizing the PCR products, we determined that all of the individual clones contained the expected ~3.6-kb RdRP-specific fragment; however, in each sequence, an average of eight nucleotide substitutions was detectable (data not shown). Most of these EuroTaq polymerase errors led to amino acid changes. Therefore, PCR was repeated with the Expand high-fidelity PCR system under standard conditions (see Methods). Although an average error of two nucleotide substitutions was found within each of the 3.6-kb fragments, one clone was isolated that comprised the entire RdRP-specific ORF without any amino acid exchange (Figure 2B).

To verify the sequences of the cDNA clones and of the PCR products, two genomic libraries were screened with the RdRP24 and the $RdRP_{5'\ RACE}$ fragments, respectively. More than 50 hybridizing phages covering the entire coding region of the RdRP ORF were isolated, and the inserts of the excised plasmids were sequenced. By multiple sequence alignment of the exon, the PCR product, and the cDNA sequences, we confirmed that the original RdRP-specific cDNA sequence is correct (Figure 2).

The *RdRP* Gene Is a Single-Copy Gene in the Tomato Genome

The restriction sites that are indicated on the map in Figure 3 have been deduced from the sequences of cDNA and genomic clones. Within the 9-kb region of the tomato *RdRP* gene, no EcoRI site was found. In contrast, three SacI sites and two sites each of BglII, XbaI, and SphI have been mapped. DNA gel blot analysis of tomato genomic DNA that had been cut by the five enzymes was performed to confirm the sequencing data and to determine the copy number of the tomato *RdRP* gene (Figure 3).

The banding pattern of the SacI-digested DNA corresponded to the calculated fragment lengths of 0.56 and 7 kb



B

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M G K T I Q V F G F P Y L L S A E V V K   20
S F L E K Y T G Y G T V C A L E V K Q S   40
K G G S R A F A K V Q F A D N I S A D K   60
I I T L A N N R L Y F G S S Y L K A W E   80
M K T D I V Q L R A Y V D Q M D G I T L  100
N F G C Q I S D D K F A V L G S T E V S  120
I Q F G I G L K K F F F F L S S G S A D  140
Y K L Q L S Y E N I W Q V V L H R P Y G  160
Q N A Q F L L I Q L F G A P R I Y K R L  180
E N S C Y S F F K E T P D D Q W V R T T  200
D F P P S W I G L S S S L C L Q F R R G  220
V R L P N F E E S F F H Y A E R E N N I  240
T L Q T G F T F F V S Q K S A L V P N V  260
Q P P E G I S I P Y K I L F K I S S L V  280
Q H G C I P G P A L N V Y F F R L V D P  300
R R R N V A C I E H A L E K L Y Y I K E  320
C C Y D P V R W L T E Q Y D G Y L K G R  340
Q P P K S P S I T L D D G L V Y V R R V  360
L V T P C K V Y F C G P E V N V S N R V  380
L R N Y S E D I D N F L R V S F V D E E  400
W E K L Y S T D L L P K A S T G S G V R  420
T N I Y E R I L S T L R K G F V I G D K  440
K F E F L A F S S S Q L R D N S V W M F  460
A S R P G L T A N D I R A W M G D F S Q  480
I K N V A K Y A A R L G Q S F G S S R E  500
T L S V L R H E I E V I P D V K V H G T  520
S Y V F S D G I G K I S G D F A H R V A  540
S1K C G L Q Y T P S A F Q I R Y G G Y K  560
G V V G V D P D S S M K L S L R K S M S  580
K Y E S D N I K L D V L G W S K Y Q P C  600
Y L N R Q L I T L L S T L G V K D E V L  620
E Q K Q K E A V D Q L D A I L H D S L2K  640
A Q E A L E L M S P G E N T N I L3K A M  660
L N C G Y K P D A E P F L S M M L Q T F  680
R A S K L L D L R T R S R I F I P N G R  700
T M M G C L D E S R T L E Y G Q V F V Q  720
F T G A G H G E F S D D L H P F N N S R  740
S T N S N F I L K G N V V V A K N P C L  760
H P G D I R V L K A V N V R A L H H M V  780
D C V V F P Q K G K R P H P N E C S G S  800
D L D G D I Y F V C W D Q D M I P P R Q  820
V Q P M E Y P P A P S I Q L D H D V T I  840
E E V E E Y F T N Y I V N D S L G I I A  860
N A H V V F A D R E P D M A M S D P C K  880
K L A E L F S I A V D F P K T G V P A E  900
I P S Q L R P K E Y P D F M D K P D4K T  920
S Y I S E R V I G K L F R K V K D K A P  940
Q A S S I A T F T R D V A R R S Y D A D  960
M E V D G F E D Y I D E A F D Y K T E Y  980
D N K L G N L M D Y Y G I K T E A E I L 1000
S G G I M K A S K T F D R R K D A E A I 1020
S V A V R A L R K E A R A W F K R R N D 1040
I D D M L P K A S A W Y H V T Y H P T Y 1060
W G C Y N Q G L K R A H F I S F P W C V 1080
Y D Q L I Q I K K D K A R N R P V L N L 1100
S S L R A Q L S H R L V L K *           1114
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Figure 2. Structure of the Tomato *RdRP* Gene and the RdRP Protein.

(A) Schematic representation of the physical structure of the tomato *RdRP* gene. The diagram represents the *RdRP* transcript, with four introns indicated by lines angled into V's. The initiation (ATG) and termination (TGA) codons are indicated. The locations of the RdRP15_H1 and RdRP8_R1 primers (Figure 1, primer pair B) are shown. A continuous cDNA clone was isolated after PCR with tomato cDNA by using the P127BamI and P127Bgl primers (see Methods). The nucleotide sequence of the C-RdRP cDNA has the accession number Y10403 in the EMBL and GenBank nucleotide sequence databases.

(B) Predicted amino acid sequence of the RdRP cDNA. The microsequenced peptides are numbered (1 to 4), and their respective amino acid sequences are printed in boldface. The peptide sequences printed in italic were not used for primer design. The first RdRP-specific PCR product ($RdRP_{800}$) that was amplified with primer pair B (see **[A]**) is underlined. The stop codon is marked with an asterisk.

(Figure 3, lane 1). The length of the latter fragment confirms the existence and the lengths of introns 1 to 4 that have been detected on genomic clones. Interestingly, intron 1 of ~1.3 kb is located 65 bp upstream of the AUG in the 5′ untranslated region. The two additional fragments of 5.5 and 9 kb represent the 5′ and 3′ border fragments of the *RdRP* gene. The length of the smaller BglII fragment (Figure 3, lane 2) is identical to the length of 1.4 kb that was determined by sequence analysis. The 5′ BglII site is located in the 480-bp-long intron 4, whereas the 3′ BglII site was found in the putative *RdRP* gene terminator. The XbaI-restricted DNA resulted in three hybridizing fragments (Figure 3, lane 4). The 630-bp fragment comprises the junction between the 5′ translated region and the 2.4-kb intron 2. The two other bands again represent the 5′ and 3′ border fragments, respectively.

According to the BglII-XbaI double digest (Figure 3, lane 3), the 7.5- and 2.4-kb XbaI fragments (Figure 3, lane 4) could be assigned to the 3′ and 5′ borders, respectively. As expected from the sequencing data, the entire tomato *RdRP* gene could be released by EcoRI (Figure 3, lane 5). Because the two SphI sites that are located within the *RdRP* gene are separated by 11 bp, only two bands are visible in the EcoRI-SphI double digest (Figure 3, lane 6). Differences in the intensity of the hybridizing fragments are based on the fact that the α-^{32}P-dCTP–labeled DNA probe comprised the RdRP-specific cDNA but not the intron sequences. Thus, fragments containing large parts of the introns (see Figure 3, map) gave relatively weak signals.

Identification of RdRP Homologs in Different Plant Species

HindIII-restricted human genomic DNA and that from potato, Arabidopsis, tobacco, and the two tomato cultivars Rentita and St. Pierre were analyzed by DNA gel blot hybridization by using a 1.7-kb 3′-specific probe (Figure 4). Compared with the identical banding patterns of the two tomato DNAs, a HindIII-specific restriction fragment length polymorphism was found for the strongly hybridizing potato DNA and for the tobacco DNA. The barely visible signals of the tobacco fragments became more distinct on an autoradiograph that was exposed for 72 hr (Figure 4, lane Tobacco*); however, in neither the human nor Arabidopsis DNA was a hybridizing fragment detectable on the 72-hr-exposed autoradiograph (data not shown).

To find conserved sequences within the *RdRP* genes of different plant species, we performed PCR amplification

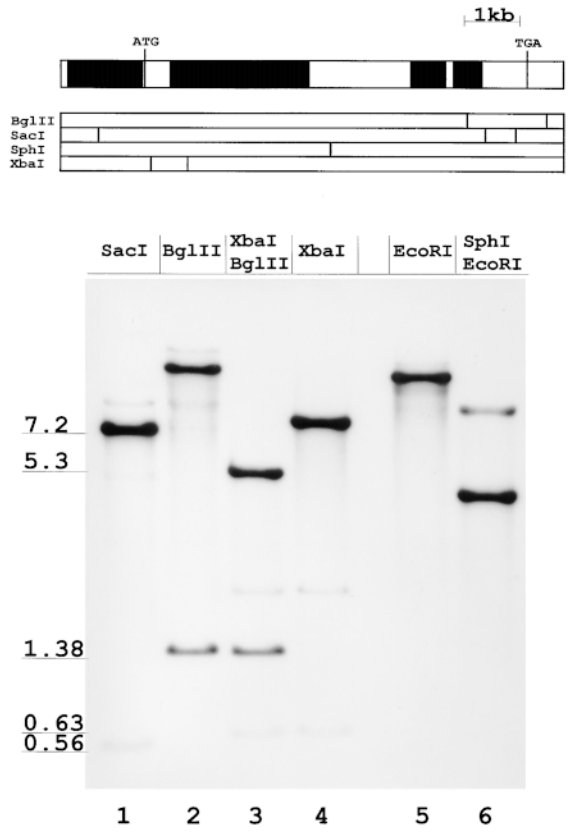

Figure 3. DNA Gel Blot Analysis of Total Genomic Tomato DNA.

On the physical map of the *RdRP* gene at top, the four introns are indicated by black boxes. The initiation (ATG) and termination (TGA) codons are indicated. The positions of sites recognized by the restriction endonucleases used in the gel blot (bottom) are shown by vertical bars in the schematic (middle). The lengths of hybridizing DNA fragments are presented in kilobases to the left of the gel. The DNA gel blot was obtained with total genomic tomato DNA that was hybridized with the entire α-^{32}P-dCTP–labeled RdRP-specific cDNA and autoradiographed for 16 hr.

with tomato (as a control) and tobacco genomic DNA by using five C-RdRP–specific primer pairs (sequences not shown). The PCR products were hybridized using the entire tomato *RdRP* sequence as a probe. One tobacco-specific product of ~500 bp clearly hybridized with the tomato-specific probe. Characterization of this cloned fragment ($RdgTb_{500}$) revealed a nucleic acid sequence identity of ~91.6% with the tomato *RdRP* coding region (data not shown). To verify that the mismatches were not due to EuroTaq polymerase or sequencing errors, we repeated PCR amplification; however, tobacco cDNA was used Fas a template. The forward primer was deduced from the $RdgTb_{500}$ sequence, and a $p(dT)_{15}$ oligomer served as reverse primer. By analyzing the cloned ~950-bp PCR product ($RdcTb_{950}$), we confirmed the $RdgTb_{500}$ sequence. The nucleic acid sequence identity between $RdcTb_{950}$ and the tomato cDNA was 89.7% within the 810-bp-long part of the coding region. In the 3′ untranslated region, identity decreased to 55.6% (data not shown). For the protein, a similarity of 96.7% and an identity of 88.2% were determined for the 270 amino acids.

Based on these results, two forward and two reverse primers corresponding to conserved regions of the tomato and the tobacco cDNA were designed. Using these primers, we amplified a 540-bp intron-free PCR product ($RdgPt_{500}$) from genomic DNA of petunia. Sequence analysis revealed 90.81% nucleic acid sequence identity between $RdgPt_{500}$ and the tomato cDNA and 90.63% identity between the petunia-specific DNA and the $RdcTb_{950}$. For the protein, similarity of 99.45% and identity of 87.3% were determined between the petunia and the tomato sequences, whereas similarity of 98.34% and identity of 88.96% were calculated for the 181 amino acids of the petunia and tobacco clones.

Alignment of the tomato-, tobacco-, and petunia-specific sequences revealed several highly conserved amino acid regions. Two forward and two reverse degenerate primers were used in PCRs with Arabidopsis and with wheat genomic

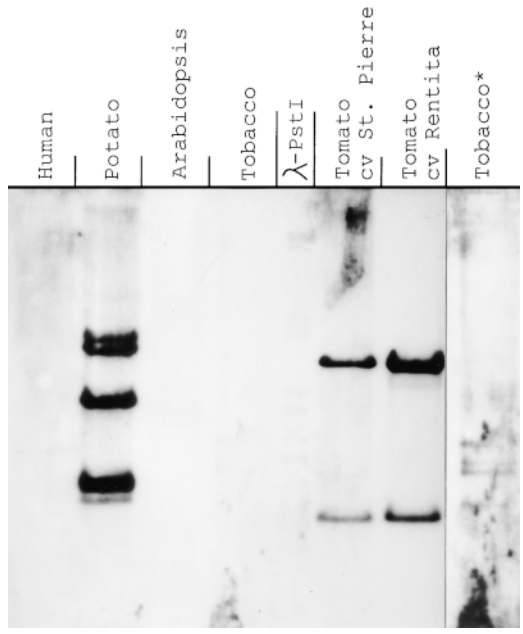

Figure 4. DNA Gel Blot Analysis of Total Genomic DNA from Different Plant Species and Humans.

The DNA gel blot was obtained with HindIII-restricted total genomic DNAs from the indicated organisms that were hybridized with a 1.7-kb α-^{32}P-dCTP–labeled 3′-specific RdRP cDNA probe and autoradiographed for 16 hr. The filter was reexposed for 72 hr to increase the signal strength of the tobacco-specific fragments (lane Tobacco*). On the overexposed autoradiograph, no hybridizing fragments were visible in the human and Arabidopsis lanes. Thus, only the Tobacco* lane was included. λ-PstI, phage lambda DNA restricted with PstI.

DNAs. Amplified products were characterized, and autotranslation of the nucleic acid sequence indicated that both Arabidopsis and wheat also contained a C-RdRP homolog. The different RdRP-specific sequences amplified from Arabidopsis, wheat, petunia, and tobacco overlapped across a 93–amino acid region. Sequence alignment of this C-RdRP region with that of the other plant species is presented in Figure 5. This analysis reveals a high level of identity between the different plant sequences and the predicted C-RdRP amino acid sequence, ranging from 72% for the Arabidopsis sequence to ~88% for the petunia and tobacco sequences (see also above). The identity between the tomato and the Arabidopsis as well as between the tomato and the wheat coding sequences is ~70%. This degree of sequence divergence would explain the failure to detect the Arabidopsis *RdRP* gene on the DNA gel blot (Figure 4) with the tomato-specific probe.

Association of T-RdRP Activity with the cDNA-Encoded 127-kD Protein

To show directly that the C-RdRP and the T-RdRP are identical, we introduced RdRP cDNA constucts into *Escherichia coli* to express a functional enzyme. Unfortunately, it turned out that the *E. coli*–synthesized C-RdRP was deposited as an insoluble and inactive protein in bacterial inclusion bodies (data not shown). Refolding of the denatured C-RdRP also failed to recover enzyme activity.

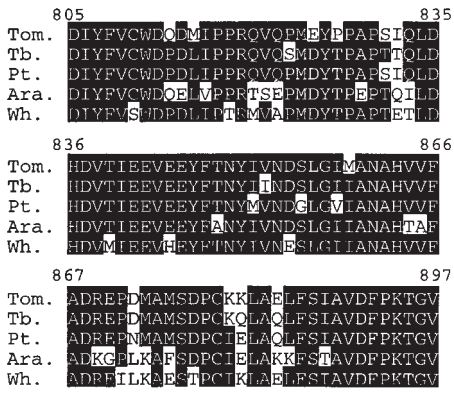

Figure 5. Partial Comparison of the C-RdRP Amino Acid Sequence with That of Tobacco, Petunia, Arabidopsis, and Wheat.

Putative RdRP sequences from other plant species were amplified by PCR by using different combinations of RdRP-specific primers (see the text). The deduced amino acid sequences from tobacco (Tb.), petunia (Pt.), Arabidopsis (Ara.), and wheat (Wh.) RdRP-specific PCR products were aligned with the predicted tomato (Tom.) RdRP amino acid sequence. Identical amino acid residues are shaded black. The sequence alignment is given from positions 805 to 897, according to the C-RdRP amino acid sequence.

Thus, to demonstrate the identity of the T-RdRP with the C-RdRP, we examined whether T-RdRP activity was consistently associated with the C-RdRP protein assayed with antisera to C-RdRP peptides (see Methods) during the five purification steps. As source material, we used the 30,000*g* pellet of a homogenate that was extracted from 220 g of viroid-infected apical tomato leaves. The pellet, containing only the minor portion of total T-RdRP, was used because it was shown to be less contaminated by terminal nucleotidyl transferase(s) (TNTase[s]) than was the 30,000*g* supernatant. TNTase activity distorts RdRP activity values that are determined by the standard assay (Schiebel et al., 1993a).

The relative enrichment of T-RdRP protein and the association of increasing specific RdRP activity with the degree of purity during purification are documented in Figure 6. The solubilized pellet (Figure 6A, lane 1) was applied using Q-Sepharose Fast Flow chromatography (Figure 6A, lane 2), which was followed by a Q-Sepharose high-performance purification step (Figure 6A, lane 3). These two anion exchange chromatography steps removed the majority of protein, including the TNTase(s). The most effective increase in specific activity was achieved by the polynucleotide affinity media poly(A)– and poly(U)–Sepharose. The T-RdRP fractions from these two columns contained low amounts of other proteins (Figure 6A, lanes 4 and 5). The purest enzyme could be eluted from a hydroxyapatite column, with only a single band visible on the silver-stained gel (Figure 6A, lane 6). However, hydroxyapatite chromatography was not performed for large-scale preparation of active T-RdRP. For reasons unknown, we recovered only about half of the enzyme activity that was applied to this column.

The identity of the T-RdRP with the C-RdRP was demonstrated by an immunoblot (Figure 6B) that was comparable to the silver-stained gel. Only one protein of ~127 kD was detectable by the C-RdRP–specific antibody A_{P431} (see Methods). In addition, the intensity of the immunoresponse increased with respect to the rise in the specific RdRP activity (Figure 6B). The additional signals on the protein gel blot (Figure 6B, lanes 1 to 3) that correspond to proteins of ~80 and 50 kD, respectively, were also detectable with preimmune serum (data not shown), pointing to contamination of the A_{P431} immune serum with non-C-RdRP–specific antibodies. Enzyme activity of the hydroxyapatite eluates was lower than expected from the signal strength of the silver-stained gel and the protein gel blot. However, the eluate in the sixth lane (Figures 6A and 6B) is presented to show the homogeneity of the preparation.

Coincidence of the C-RdRP with the activity of the T-RdRP is further demonstrated in Figures 7A and 7B by immunoblots showing eluted fractions from poly(U)–Sepharose and hydroxyapatite chromatography. On the gels that are depicted in Figure 6, the highest RdRP activity–containing fractions that were eluted from each of the five columns have been electrophoresed. In contrast, Figures 7A and 7B reflect parts of elution profiles. The increase and decrease of

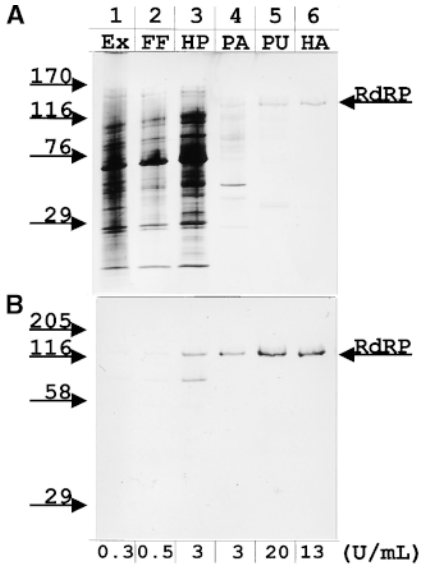

Figure 6. SDS-PAGE Analysis of the Purification Procedure of T-RdRP from the 30,000*g* Pellet of a Leaf Homogenate.

(A) Silver-stained protein pattern of RdRP preparations.
(B) Protein gel blot corresponding to **(A)** and visualizing the T-RdRP by the C-RdRP–specific antibody A_{P431}.
The positions of molecular mass markers are indicated in kilodaltons at left. The specific RdRP activity that was determined for each fraction is given in units (U) per milliliter. The volume of samples loaded onto the gels was 1 μL per lane. Lane 1, combined extracts from the 30,000*g* pellets (Ex); lane 2, Q-Sepharose Fast Flow eluate (FF); lane 3, Q-Sepharose high-performance eluate (HP); lane 4, poly(A)–Sepharose eluate (PA); lane 5, poly(U)–Sepharose eluate (PU); and lane 6, hydroxyapatite eluate (HA). The arrows at right denote the 127- kD band of the RdRP, and those at left indicate the positions of molecular weight markers in kD. Experimental details are given in Methods.

enzyme activity during the T-RdRP elution from both columns were in perfect accordance with the immunoresponse to A_{P431}. The intensity of coloration of the protein gel blot is proportional to the T-RdRP activity values.

Finally, the association of the C-RdRP with the RdRP activity is documented by the anomalous gel filtration behavior of T-RdRP on Superdex 200 that is illustrated in Figure 8. An eluate from the hydroxyapatite chromatography was supplemented with rabbit IgG (150 kD) and BSA (66 kD). The protein mixture was chromatographed, and a comparison of the UV_{280} values with the RdRP activity profile of the eluted fractions led us to believe that the T-RdRP is smaller than the BSA protein. According to size, the T-RdRP was expected to be eluted very soon after the IgG marker protein and clearly before a 66-kD protein. However, elution volumes of 1.35 mL for IgG, 1.50 mL for BSA, and 1.58 mL for the T-RdRP were determined (Figure 8). After SDS gel analysis of the UV_{280}-absorbing material, we found that the prominent protein of the RdRP activity–containing fraction was 127 kD (data not shown). The identity of this 127-kD protein with the C-RdRP was demonstrated with A_{P431} (Figure 8, inset gel).

Induction of T-RdRP Activity upon Viroid Infection Is Correlated with an Increased Steady State Level of RdRP mRNA

Upon RNA virus and viroid infection, plant-encoded RdRPs increase both in activity and in amount (Astier-Manifacier and Cornuet, 1971; Duda et al., 1973; Takanami and Fraenkel-Conrat, 1982; van der Meer et al., 1984; Schiebel et al., 1993a). RNA gel blot analysis was performed with total RNA isolated from potato spindle tuber viroid (PSTVd)–infected and viroid-free tomato plants. Hybridization with an α-^{32}P-dCTP–labeled RdRP-specific DNA probe revealed a three- to fivefold increase in the amount of steady state RdRP mRNA in the two PSTVd-infected tomato cultivars Rutgers and Basket Pak (Figure 9; cf. lanes 1 and 3 with lanes 2 and 4). The increased RdRP activity of approximately threefold, which was previously reported for systemically PSTVd-infected tomato leaves (Schiebel et al., 1993a), is in perfect agreement with the results obtained by the RNA gel blot analysis. This indicates that in viroid-infected tomato plants, RdRP induction is due to an enhancement of transcription and/or mRNA stability.

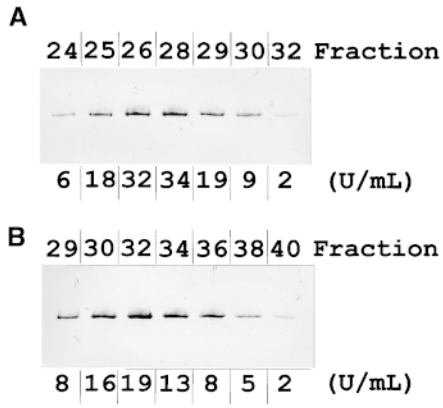

Figure 7. RdRP Activity Coincides with an A_{P341}-Detectable Tomato-Specific 127-kD Protein in Eluates from Poly(U)–Sepharose and from Hydroxyapatite.

(A) Protein gel blot analysis of Poly(U)–Sepharose eluates with the C-RdRP–specific antibody A_{P431}. The staining intensity of the 127-kD band strongly correlates with the activity of RdRP. Samples were from 100-μL fractions of poly(U)–Sepharose chromatograph eluates (see Figure 6). U, units.
(B) Protein gel blot analysis as shown in **(A)**, but samples were from the hydroxyapatite eluate fractions (see Figure 6).

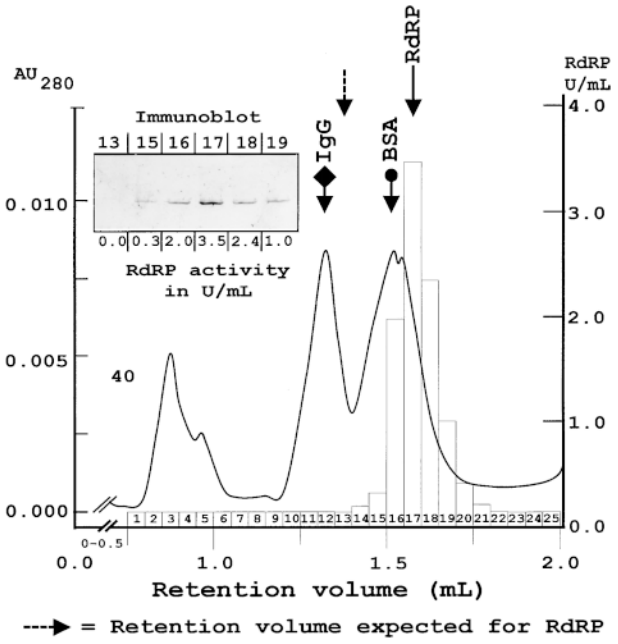

Figure 8. Delayed Coelution of T-RdRP from a Superdex 200 Column.

Eluate from the hydroxyapatite chromatography representing ~0.75 units of T-RdRP in 15 μL of 0.16 M NaPi in buffer F was supplemented with 2 μg each of rabbit IgG (Sigma) and BSA (Serva 11924) and loaded onto a precalibrated Superdex 200 column in the Smart system. The column was equilibrated at 5°C with a buffer of 0.15 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1.5 mM DTT. Elution volumes were 1.35 mL for IgG, 1.505 mL for BSA, and 1.58 mL for RdRP. Fractions containing enzyme activity were subjected to protein gel blot analysis with the C-RdRP–specific antibody A_{P431}. The immunoblot shows a single band of 127 kD and the maximum of staining intensity in fraction 17. Neither RdRP activity nor a 127-kD protein was detectable in fraction 13, in which a standard protein of this size should elute. AU, absorbance units; U, units.

In Vitro Analysis of the T-RdRP

If plant-specific RdRPs are the key enzymes in post-transcriptional gene silencing processes, then they should be capable of synthesizing cRNAs of ~50 to 130 nucleotides from RNA templates. To examine the in vitro T-RdRP–synthesized products, we modified the RdRP activity standard assay (see Methods). Total tomato RNA was used as template, and the products were analyzed by using PAGE.

RNA fragments of >100 nucleotides were produced when the assay was performed with 1 μg of template RNA (Figure 10, lane 2). A decrease in the template (0.1 μg) also resulted in a decrease in products. On the other hand, increased amounts of total RNA (5 μg) had little effect on product amount and quality (Figure 10, lanes 1 to 3). The capability of the T-RdRP to also use DNA as template appeared to be very low. The standard assay was performed with total genomic tomato DNA as well as with linearized plasmid DNA, but only a few products were detectable after PAGE (Figure 10, lanes 5 to 7). To demonstrate that the products consisted of newly synthesized RNA and were not due to a T-RdRP–specific terminal transferase activity (Schiebel et al., 1993b), the nucleotide triphosphates were left out in one reaction. Because no product was visible, it can be concluded that the T-RdRP is not able to initiate the addition of at least α-^{32}P-UTP (see Methods) to the template RNA (Figure 10, lane 8). The identity of RNA as the T-RdRP–synthesized product was further investigated by treating the reaction mix with RNase A before PAGE (Figure 10, lane 9). In addition, it was shown that the template is destroyed by RNase A but not by DNase treatment (data not shown), indicating that RNA is the real substrate of the T-RdRP.

DISCUSSION

Analysis and characterization of the in vitro and in vivo properties of plant RdRPs require suitable amounts of pure enzyme. To obtain increased RdRP activity, researchers have almost always used RdRP purification protocols that begin with RNA virus–infected plants. But despite many attempts to clarify the nature of the host-encoded RdRP (reviewed in

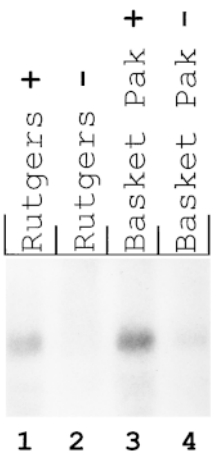

Figure 9. RNA Gel Blot Analysis of Total RNA from Viroid-Infected and Viroid-Free Tomato Plants.

The steady state level of RdRP-specific mRNA was examined in viroid-infected (+) and in viroid-free (−) tomato plants. The same amounts (15 μg per lane) of total leaf RNA from infected as well as from noninfected tomato cultivars Rutgers and Basket Pak were hybridized with a 1.7-kb α-^{32}P-dCTP–labeled 3′-specific RdRP cDNA probe and autoradiographed for 16 hr.

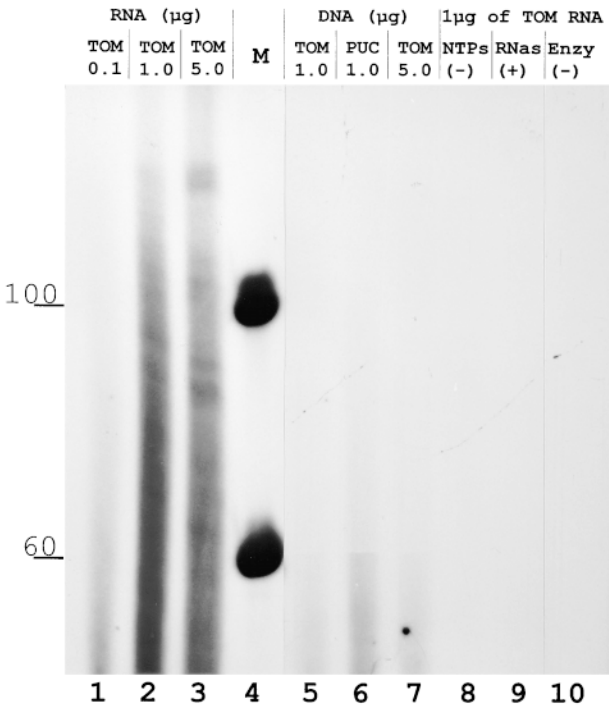

Figure 10. Analysis of in Vitro–Synthesized T-RdRP Products by Using PAGE.

RdRP activity standard assay samples were separated on a 10% polyacrylamide gel to analyze the amount and quality of the RdRP-synthesized products. Substrate saturation was examined by using 0.1, 1.0, and 5.0 µg of total tomato (TOM) RNA as template (lanes 1 to 3), and the suitability of DNA to serve as template was analyzed by performing the standard assay with 1.0 and 5.0 µg of total tomato DNA as well as with 1.0 µg of linearized plasmid DNA (PUC) (lanes 5 to 7). As a control, the assay was performed in the absence (−) of nucleotide triphosphates (NTPs [−], lane 8) and in the absence of enzyme (Enzy [−], lane 10). The identity of the RNA nature of the RdRP-synthesized products was confirmed by treating the sample with RNase A (RNas [+], lane 9) before PAGE. The lengths of the in vitro–radiolabeled RNA products used as size markers (M, lane 4) are presented in nucleotides to the left of the gel.

Fraenkel-Conrat, 1986), the activity of the isolated enzyme(s) could not be clearly assigned to the plant- and virus-encoded RdRP (Khan et al., 1986). Thus, from cowpea mosaic virus (CPMV)–infected plant tissue, for example, two RdRP activities were obtained, one of which was assumed to represent the host-encoded RdRP, whereas the other was considered to be specific for CPMV-infected leaves (Dorssers et al., 1983). But apart from our studies with the T-RdRP (Schiebel et al., 1993a, 1993b), no detailed characterization of an RdRP from other plant species has been reported so far.

Microsequencing of the Enzyme and Isolation of Its cDNA

Endoproteolytic digestion of the gel-excised 127-kD protein(s) resulted in ~50 reversed-phase HPLC–specific peaks. Most of these contained a mixture of several protein degradation products. Nevertheless, four peptide sequences were obtained from which PCR primers were designed. By using a combination of two of these primers, the $RdRP_{800}$ fragment was amplified, which finally allowed isolation of the entire C-RdRP cDNA. The fact that the peptide sequences deduced from the other two primer sequences could be found within the protein sequence of the C-RdRP substantiated that the isolated cDNA is coding for the microsequenced protein. Because an active C-RdRP protein from *E. coli* cells was not expressed, we analyzed indirectly the identity of the C-RdRP with the help of antibodies that had been raised against synthetic C-RdRP–specific peptides. The four antibody preparations specifically detected a 127-kD tomato protein in an aliquot of the protein fraction that was applied during the microsequencing procedure. As a control, the most sensitive A_{P431} immune serum, which has been chosen for further experiments, was demonstrated also to bind to the *E. coli*–expressed C-RdRP (data not shown). It is noteworthy that immunoprecipitation experiments with A_{P431} led to inhibition of T-RdRP activity, but splitting off the antibody from the protein did not restore activity (data not shown).

Because the A_{P431} immunoresponse perfectly correlated with the enzyme activity during all enzyme purification steps, we assumed that the C-RdRP and the T-RdRP are identical. However, the remote possibility exists that at least two proteins might have comigrated and become excised together from the SDS gel and also applied together to the microsequencing procedure. If so, this would mean that the contaminating protein shares those physical properties that have rendered possible the purification of the T-RdRP up to a single band in a silver-stained SDS gel. In addition, the observation that the A_{P431} immunoresponse also correlated with the unusual behavior of the T-RdRP in the Superdex 200 gel filtration precluded the presence of a second protein. All of these data strongly suggest that the microsequenced protein is the actual T-RdRP and that the isolated cDNA represents its genomic transcript.

Cellular RdRPs in Other Plant Species

The availability of a full-length tomato RdRP cDNA facilitated the search for the presence of this enzyme in other plant species. The existence of highly conserved *RdRP* genes in potato, tobacco, wheat, Arabidopsis, and petunia could be clearly shown by DNA gel blot hybridization and/or PCR amplification experiments. The failure to detect a corresponding sequence in Arabidopsis by DNA gel blot hybridization of genomic DNA against the tomato-specific probe demonstrated the limitation of this procedure. The

fact that an *RdRP*-specific fragment from Arabidopsis could be amplified by PCR has shown that the similarity between the T-RdRP and the RdRP of Arabidopsis was too low for cross-hybridization under the applied conditions. It should be mentioned that an expressed sequence tag from Arabidopsis (EMBL accession number B61313) of ~0.55 kb perfectly matched our Arabidopsis PCR product. Also, of direct relevance to our findings are the studies of a putative cowpea-specific RdRP cDNA fragment (sequence according to S. Rudd, Norwich, UK). This sequence shows 73% nucleotide sequence identity and 95% amino acid sequence similarity with the T-RdRP cDNA.

Cellular RdRPs in Other Organisms and Systems

Our search of RdRP sequences in other organisms and systems has shown that ORFs of *S. pombe* (EMBL accession number Z98533) and *C. elegans* (EMBL accession number Z48334) most likely encode an RdRP homolog. Both of the amino acid sequences contain several regions that are identical or highly similar to that of the plant RdRPs. However, no evidence for a human RdRP has as yet been found. Interestingly, plant RdRPs evidently do not share any similarity to the known virus RNA replicases, including in their common GDD sequence motif (Jablonski et al., 1993; Routhier and Bruenn, 1998). Future PCR experiments with degenerate primers deduced from peptides that are conserved in the tomato, tobacco, petunia, Arabidopsis, wheat, yeast, and nematode amino acid sequences will help to extend our knowledge on the still largely enigmatic cellular RdRP enzyme.

Possible Functions of the Cellular RdRP

The involvement of a plant-encoded RdRP in RNA-mediated gene silencing and in RNA-mediated virus resistance is one of the most crucial points of the current models for homology-dependent gene silencing (reviewed in Wassenegger and Pélissier, 1998). In a series of experiments on transgene-induced silencing, it has been suggested that RNA most likely mediates post-transcriptional gene silencing. By using grafting experiments, it has been demonstrated that the effector of post-transcriptional gene silencing can be transmitted systemically. Transgene-specific silencing was unidirectionally transmitted from silenced stocks to nonsilenced scions (Palauqui et al., 1997). It was further observed that post-transcriptional cosuppression of the tobacco nitrate reductase (*Nia*) genes and the *Nia*2 transgene is dependent on the transcriptionally active state of the transgene (Vaucheret et al., 1997). Similar results were obtained by analyzing the frequency and degree of cosuppression by sense chalcone synthase transgenes in petunia. The finding that cosuppression was dependent on transgene promoter strength and was reduced by premature nonsense codons in the transgene coding sequence led to the conclusion that transgene transcription is necessary for induction of post-transcriptional gene silencing by single-copy transgenes (Que et al., 1997).

If this is true, how can a primary transcript be enabled to decrease the steady state mRNA of the corresponding gene? It is generally assumed that short cRNA molecules are transcribed from sense transcripts by a cellular RdRP. Subsequently, the cRNAs could specifically target mRNA degradation. However, as of yet, there is no proof of such a mechanism. Moreover, almost no alternatives have been presented by which this highly specific cytoplasmic RNA/RNA interaction–dependent process could be replaced. Nevertheless, it should be noted that RdRP-independent mechanisms of RNA degradation have been proposed (Cameron and Jennings, 1991; Metzlaff et al., 1997) that involve intermolecular interactions between sense transcripts and recognition of paired complementary sequences by dsRNA-specific RNases.

When we examined the existing models, we found that the T-RdRP could meet all of the requirements of the proposed cRNA-involving mechanism. The analysis of the catalytic in vitro properties of T-RdRP revealed that it accepts single-stranded RNAs as templates and, regardless of whether these RNAs are primed or unprimed, cRNAs are synthesized (Schiebel et al., 1993b). Although these experiments were performed predominantly with 12- to 14-nucleotide-long synthetic RNA or DNA oligomers, T-RdRP–produced cRNAs can have a length of >100 nucleotides. This was shown by using an in vitro transcript of the P35S (+) strand (data not shown) and total tomato leaf RNA. Interestingly, for post-transcriptional gene silencing, a cRNA size ranging between 10 and 75 nucleotides was proposed to be sufficient to target RNA degradation efficiently (Dougherty and Parks, 1995). In addition, homology of 60 to 130 bp between an inactivating transgene and a target sequence has been shown to be sufficient to trigger post-transcriptional gene silencing and RNA-mediated virus resistance (Cogoni et al., 1996; Sijen et al., 1996; Pang et al., 1997).

According to the threshold hypothesis originally proposed by Lindbo et al. (1993), overexpressed mRNA accumulating in the cytoplasm above an aberrant level could be used by a host RdRP for the production of the cRNAs, which would activate the silencing process (Lindbo et al., 1993; Dougherty and Parks, 1995). This hypothesis can explain silencing by single-copy, highly transcribed transgenes but does not readily explain those examples in which gene silencing is triggered with inverted repeat transgenes driven by weak promoters (Que et al., 1997; Stam et al., 1997b). Other studies also reported examples of silenced and nonsilenced transgenic lines that display no detectable difference in the transgene transcription rate (Mueller et al., 1995; English et al., 1996). These results strengthen the emerging view that not only quantitative but also qualitative features (e.g., "aberration" in the structure and/or the location of a transcript) of the (trans)gene mRNAs could define these molecules as

preferred templates for the host RdRP, resulting in the activation of the post-transcriptional gene silencing process (English et al., 1996; reviewed in Baulcombe, 1996; Sänger et al., 1996; Depicker and Van Montagu, 1997; Stam et al., 1997a; Wassenegger and Pélissier, 1998).

In both the threshold and the aberrant RNA models, it was suggested that the involvement of RdRP-produced cRNAs accounts for the high sequence specificity of gene silencing. Therefore, this might indicate that there are at least two ways to trigger the RdRP-dependent degradation mechanism. It is also possible that a threshold level of aberrant RNA is required in all cases to initiate the silencing process. In many transformation experiments, it was observed that single copies rarely gave rise to silencing, whereas inverted repeats of the transgene at a single locus frequently initiated silencing (Hobbs et al., 1993; van Blokland et al., 1994; English et al., 1996; Que et al., 1997; Stam et al., 1997b). This might reflect the potentiality of inverted repeats to produce more aberrant RNA than do single-copy transgenes (Que et al., 1997) and/or that aberrant RNA produced by inverted repeats represents a more efficient substrate for the RdRP than the one produced by single-copy transgenes (Wassenegger and Pélissier, 1998). Future in vitro analysis of T-RdRP substrate specificity might allow us to define some sequence and/or structural motifs that could tag RNA molecules as efficient templates for the RdRP.

Interestingly, in viroid-infected tomato plants, enhanced RdRP activity correlates with an increased steady state level of the RdRP mRNA. Viroids are small, pathogenic, circular RNA molecules that display a stable, rodlike structure (i.e., a highly double-stranded form). They are capable of autonomous replication in the nucleus of the host cell, where they can accumulate up to 5×10^4 copies (Harders et al., 1989). Thus, if the viroid RNA can be considered as a kind of aberrant RNA, then this result might indicate that *RdRP* gene expression and consequently RdRP activity could be, at least to some extent, regulated by nuclear concentrations of aberrant RNAs. On the other hand, one can speculate that cellular RdRPs are involved in a defense mechanism that is targeted against overexpressed foreign RNAs.

Future Perspectives

Even if the T-RdRP and the C-RdRP represent identical proteins, the actual biological function of plant-encoded RdRPs has to be examined. The refined T-RdRP purification procedure will provide sufficient amounts of pure enzyme to analyze RdRP substrate specificity. The availability of the full-length tomato RdRP cDNA will facilitate the analysis of its possible involvement in homology-dependent gene-silencing phenomena. Thus, experiments allowing overexpression as well as downregulation of the enzyme in transgenic plants could help to determine whether RdRPs play some role in normal plant gene regulation or whether they are enzymes of a plant defense mechanism (Jorgensen et al., 1998).

METHODS

Isolation of RNA-Directed RNA Polymerase for Microsequencing

RNA-directed RNA polymerase (RdRP) was isolated from apical leaves (1 g per plant) of viroid-infected tomato plants (*Lycopersicon esculentum* cv Rentita), essentially as described previously (Schiebel et al., 1993a). DEAE–Sepharose was used as fast flow quality (Pharmacia Biotechnology), and the double-stranded DNA (dsDNA)–cellulose was substituted by poly(U)–Sepharose 4B (Pharmacia Biotechnology). RdRP eluted from this latter column (3 mL of gel volume) in a 20-mL linear gradient of 0 to 1.5 M NaCl in buffer A (20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1.5 mM dithioerythritol [DTE], and 0.012% Tween 20) at ~1 M salt. The enzyme was concentrated by a second round of poly(U)–Sepharose chromatography by using an HR5 column with an 0.4-mL gel volume in the Smart system (Pharmacia Biotechnology). Pooled RdRP was precipitated with trichloroacetic acid in the presence of deoxycholate and insulin (Bensadoun and Weinstein, 1976). The pellet was washed with absolute ethanol at −20°C, and the proteins were subsequently subjected to SDS-PAGE (see below).

Isolation of Enzymatically Active RdRP

Q-Sepharose Chromatography

The extracts obtained from the 30,000*g* pellet (Schiebel et al., 1993a) by centrifugation (146,000*g* for 50 min in a Beckman-type 50.2 rotor; Beckman Instruments, Inc., Fullerton, CA) were loaded with a flow rate of 30 cm/hr onto a 70-mL column of Q-Sepharose Fast Flow (Pharmacia Biotechnology) equilibrated with buffer B (25 mM Tris–acetate, pH 8.2, 1 mM EDTA, 20% glycerol, and 3 mM 2-mercaptoethanol). In a linear 195-mL gradient, ammonium acetate was raised to 0.7 M in buffer B. RdRP eluted at ~370 mM salt. The pool was diluted with two volumes of buffer B and further purified on Q-Sepharose high-performance 16/10 (Pharmacia Biotechnology) with a 58-mL salt gradient in buffer B.

Poly(A)–Sepharose Chromatography

The RdRP pool was diluted with 1 volume of buffer C (50 mM Tris-HCl, pH 7.5, 15% glycerol, 1.5 mM DTE, and 0.012% Tween 20) and loaded onto a 4-mL poly(A)–Sepharose 4B C10/10 column (Pharmacia Biotechnology). After raising NaCl to 0.1 M in buffer C, a 2-mL gradient from 0.1 to 0.3 M salt with a flow rate of 30 cm/hr was applied. RdRP eluted between 0.15 and 0.3 M salt.

Poly(U)–Sepharose Chromatography

RdRP was diluted with 1 volume of buffer C and chromatographed on 0.2 mL of poly(U)–Sepharose 4B by using an HR5 column and the Smart system (Pharmacia Biotechnology). A linear 1-mL gradient from 0.35 to 0.7 M NaCl in buffer C was applied with a flow rate of 30 cm/hr. RdRP eluted at ~0.6 M salt.

Hydroxyapatite Chromatography

RdRP was diluted with 2 volumes of buffer D (25 mM Tris–acetate, pH 8.2, 15% glycerol, 1.5 mM DTE, and 0.012% Tween 20) and chromatographed on 0.2-mL hydroxyapatite (ceramic hydroxyapatite type I, 40 μm; Bio-Rad) in the Smart system. The column was prewashed with 5 mL of 1 mM $MgCl_2$ (Gorbunoff, 1985). A concentration of 60 mM sodium phosphate, pH 6.8, in buffer D was kept for 20 gel volumes and increased in a linear 2.2-mL gradient to a 1-mL plateau of 180 mM sodium phosphate. The RdRP eluted at ~150 mM.

Superdex 200 Gel Filtration

Gel filtration was performed with a Superdex 200 PC 3.2/30 column (Pharmacia Biotechnology) by using the Smart system. The column was equilibrated at 5°C with a buffer of 0.15 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1.5 mM DTE. Rabbit IgG (Eurogentec, Seraing, Belgium) and BSA (Serva 11924; Boehringer Ingelheim Bioproducts, Heidelberg, Germany) were used as reference proteins.

Standard Assay for RdRP Activity

Enzyme activity was measured as described by Schiebel et al. (1993a) with the following modifications. The final volume of 25 μL in prelubricated 1.7-mL test tubes (Sorenson Bioscience, Inc., Salt Lake City, UT) contained 0.02 mM α-^{32}P-UTP (adjusted to 0.5 Ci/mmol) and was supplemented with 0.01% Tween 20. The reaction was stopped on ice by the addition of 15 μL of 13 mM UTP. After adding 10 μL of water, the tube contents were spotted on Whatman (Maidstone, UK) 3MM paper strips.

Protein Determination

Protein concentration was determined by using a microassay modification (Peterson, 1983) of the Coomassie Brilliant Blue R 250 dye binding method (Bradford, 1976) with 100-μL cuvettes. The concentration of purified RdRP in silver-stained polyacrylamide–SDS gels was estimated as described by Schiebel et al. (1993a).

SDS-PAGE and Protein Gel Blotting

Protein samples were prepared by boiling for 3 min in a standard reducing buffer and separated on an electrophoresis unit (Phast-System; Pharmacia Biotechnology) by using 8 to 25% Phastgels. Proteins were visualized by silver staining (Merril et al., 1981) by using a protocol for semiautomated staining (Fabri et al., 1993). Standard proteins (Pharmacia Biotechnology; high molecular weight marker SDS calibration kit) were supplemented with carbonic anhydrase (29 kD) and β-lactoglobulin (17 kD) (Sigma).

Immunoblots were obtained from Phastgels by semidry electrophoretic transfer onto nitrocellulose membrane (Protran 0.45 μm; Schleicher & Schuell). Blotted antigens were detected by an amplified alkaline phosphatase assay kit (Bio-Rad) by using affinity-purified rabbit antibody (1:500 dilution) raised against peptide P431 (see below) (Eurogentec). Biotinylated standard proteins were from Sigma (B2787).

Protein Microsequencing

The precipitated protein was prepared for SDS-PAGE, applied onto a 7% polyacrylamide gel (Laemmli, 1970), and stained with Coomassie blue (Sambrook et al., 1989). The 127-kD band was excised, and protein cleavage was performed in the gel according to Eckerskorn and Lottspeich (1989), except that instead of trypsin, endoprotease LysC (Boehringer Mannheim) with an enzyme/protein ratio of 1:10 (w/w) was used. The peptides separated by reversed-phase HPLC were sequenced using a 492A amino acid sequencer (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

Plant Material

Tobacco (*Nicotiana tabacum* cv Petit Havana SR1), petunia (*Petunia hybrida* V26), wheat (*Triticum aestivum*), and Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) were used to search for tomato RdRP (T-RdRP) homologs.

Tomato RNA Isolation, cDNA Synthesis, and Polymerase Chain Reaction

For mRNA isolation, we harvested 20 g of young leaves from potato spindle tuber viroid (PSTVd)–infected tomato plants (*L. esculentum* cv Rutgers) that were grown in the greenhouse under standard conditions. The plant material was quickly frozen in liquid nitrogen, and total RNA was extracted as described by Logemann et al. (1987). From 5 mg of total RNA, poly(A)$^+$ RNA was isolated by using Poly-A-Tract mRNA isolation system I (Promega), according to the manufacturer's instructions. cDNA synthesis was performed by using 1 μg of purified poly(A)$^+$ RNA as template (cDNA synthesis kit; Boehringer Mannheim), according to the manufacturer's instructions. The reaction was stopped by phenol extraction, and 1 μL of the sample was diluted 1:100 with Tris–EDTA buffer.

A first polymerase chain reaction (PCR) was performed with 1 μL of the diluted cDNA by using primer pairs A (RdRP_8H1-RdRP_15R1; 0.1 nmol each) and B (RdRP_15H1-RdRP_8R1; 0.1 nmol each), respectively. Amplifications were assayed in a 100-μL reaction mixture containing 10 μL of 10 × assay buffer (Eurogentec), 10 μL of deoxynucleotide triphosphates (2 nmol/μL), and 1 μL of EuroTaq polymerase (4 units per μL; Eurogentec). Thirty cycles of program 1 at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min were started. The PCR products were cloned into the T/A-type PCR cloning vector pT-PCR (Wassenegger et al., 1994). Sequences of the primers are as follows: RdRP_8H1, 5′-TAYCCNGAYTTYATGGAYAA-3′; RdRP_15H1, 5′-AARGCNCARGARGCNYTN GA-3′; RdRP_8R1, 5′-GGYTTRTCCATRAARTCNGGRTA-3′; and RdRP_15R1, 5′-ATNTTNGTRTTYTCNCCNGG3′.

A second PCR was performed with program 2 at 94°C for 1 min and 30 cycles at 94°C for 30 sec, 60°C for 30 sec, and 68°C for 4 min with 1 μL of 1:100 diluted cDNA (see above) by using the forward primer P127BamI and the reverse primer P127BgI. The sequences for these two primers are 5′-CTTCACCAGGGATCCACTCATCACTCCCCTCAAG-3′ for P127BamI and 5′-GCAGCTTCATGCAGATCTAAAGACAAAAGGTAGTC-3′ for P127BgI.

PCR, using these two primers, was repeated with the Expand high-fidelity PCR system (Boehringer Mannheim). The amplification was in a total volume of 100 μL containing 1 μL of 1:100 diluted cDNA, 10 μL of the 10 × assay buffer (adjusted to 15 mM Mg^{2+}), 1

μM of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 3.5 units of the enzyme. The sample was processed as described above (program 2).

Rapid Ampification of 5′ cDNA Ends

The rapid ampification of 5′ cDNA ends (5′ RACE) was performed by using the *RdRP* gene–specific reverse primer GSP400. Using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA), we ligated an adapter to both ends of the double-stranded cDNA. Subsequently, PCR amplification was performed with a 1:100 dilution of the cDNA (1 μL per reaction) by using the adapter sequence–specific AP1 oligonucleotide as a forward primer and GPS400 as a reverse primer. Their sequences are 5′-CATAACGAATCTGGAAAGCAGATGG-3′ for GSP400 and 5′-CCATCCTAATACGACTCACTATAGGGC-3′ for AP1. The applied thermal cycle parameters are described (program 1) above.

Screening of cDNA and Genomic Libraries

Two custom ZAP Express EcoRI cDNA libraries (Stratagene, La Jolla, CA) had been established from poly(A)$^+$ RNA, which had been isolated from young leaves of the tomato cultivars Rutgers and Basket Pak. With five to 10 μg of the purified poly(A)$^+$-enriched RNA, we constructed cDNA libraries (Stratagene). The size-fractionated oligo(dT)-primed cDNAs (>500 bp) were ligated via EcoRI adapters into the λ ZAP phage bearing the pBK-CMV phagemid vector. The Rutgers- and Basket Pak–specific cDNA libraries produced by Stratagene had a representative size of 1.8×10^6 and 3.5×10^6 plaque-forming units, respectively.

Both libraries were screened by plaque hybridization with α-^{32}P-dCTP–labeled DNA fragments by using a random primed DNA labeling kit (Boehringer Mannheim). Recombinant plasmid DNAs were excised in vivo from the phages and finally introduced into the XLOLR *Escherichia coli* strain, according to the Stratagene ZAP Express EcoRI library instruction manual.

Two representative λ ZAP Express EcoRI custom genomic libraries (Stratagene) had been established from nuclear DNA that had been isolated from 20 g of young leaves of the tomato cultivars Rutgers and Basket Pak, according to Bedbrook (1981). The size-fractionated (>1 kb) plant DNA was ligated via EcoRI adapters into the λ ZAP phage bearing the pBK-CMV phagemid vector. The Rutgers- and Basket Pak–specific genomic libraries had a representative size of 2.4×10^6 and 8.8×10^5 plaque-forming units, respectively. Both contained an estimated background of <5% nonrecombinant clones. Both libraries were screened, and recombinant plasmid DNAs were excised as described above.

DNA Sequencing and Sequence Analysis Software

All sequences were determined on an automatic sequencer (ALFexpress; Pharmacia Biotechnology) by using the Cy5 AutoRead sequencing kit (Pharmacia Biotechnology) and following the manufacturer's sequencing procedure.

DNA and amino acid sequences were analyzed by using the DNASIS for Windows program (Pharmacia Biotechnology); homology searches and sequence alignments were performed by using the Blast X and the GAP programs (version 7.0; Genetics Computer Group, Madison, WI) and the MPsrch_tpn program (Release 3.0.4D J.F. Collins).

RNA and DNA Gel Blot Analyses

Total RNA was isolated as described above from PSTVd-infected and from PSTVd-free tomato plants (cvs Rutgers and Basket Pak). Separation of total RNAs (15 μg per lane) was performed in phosphate-buffered 1.5% agarose gels. The RNAs were pretreated with 1 volume of DMSO mix in a final volume of 50 μL and heat denatured at 65°C for 10 min (Spiesmacher et al., 1985). The RNAs were transfered onto noncharged nylon membranes (Qiabrane; Qiagen, Chatsworth, CA) by capillary blotting and hybridized against random primed α-^{32}P-dCTP–labeled DNA.

DNA gel blot analysis of endonuclease-restricted genomic DNA was prepared according to Sambrook et al. (1989). The DNA was transferred (vacuum blotter; Appligene, Illkirch, France) to a positively charged nylon plus membrane (Qiagen) and finally $UV_{312\ nm}$ cross-linked (0.3 J/cm^2). Hybridization of DNA gel blots using random primed α-^{32}P-dCTP–labeled DNAs was performed as described by Amasino (1986).

Antibody Production

Four different cDNA-encoded RdRP (C-RdRP)–specific antibodies were produced (Eurogentec). From the entire amino acid sequence of the C-RdRP, the following peptides were synthesized for the immunization of rabbits: P430, SNRVLRNYSEDIDN (comprising amino acids 377 to 390); P431, ASKTFDRRKDAEAI (comprising amino acids 1007 to 1020); P432, EQYDGYLKGRQPPKSPS (comprising amino acids 331 to 347); and P433, VFPQKGKRPHNEC (comprising amino acids 784 to 796). The specific reaction of each antiserum with the T-RdRP was tested by protein gel blotting (see above). The antibody against P431 (A_{P431}) was affinity purified (Eurogentec) and used in further experiments.

Substrate Analysis by PAGE

Samples of the RdRP activity standard assay (see above) were not stopped by adding 15 μL of 13 mM UTP but were mixed with 25 μL of gel loading buffer immediately after incubation. Subsequently, the samples were separated on a 10% polyacrylamide urea gel under denaturating conditions (Sambrook et al., 1989).

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